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14. ABSTRACT
In this grant proposal, we proposed to construct Ras/Raf-based MCAMD, i.e. fuse one subunit of caspase-3 to Raf and the other subunit to Ras. Once Ras interacts with Raf in response to mitogenic signal, the two subunits of caspase-3 that are fused to Ras and Raf will be brought together to form mature active caspase-3, thus cellular apoptosis will be initiated.

During eighteen months, we constructed plasmids that are required for testing MCAMD. We characterized the interaction of separated C3SS and C3LS and established a base for application of Caspase-3 in construction of MCAMD. We tested initial cellular reactions of MCAMD and found that C3SS-Ras could not co-express with Raf-RBD-C3LS, suggesting that C3SS-Ras and Raf-RBD-C3LS might form active Caspase-3 and cause a rapid cell death. However, because this research is novel and we did not have experience in construction and testing of MCAMD, we met some unexpected problems, such as weak interaction of wild type Ras with Raf-RBD that causes constitutive activation of MCAMD and makes that MCAMD cannot sense mitogenic signaling.

Future studies will include: (a) to overcome signaling switch problem of the MCAMD; (b) to assay caspase-3 activity and apoptosis upon co-transfection of tetracycline-inducible expression of C3SS-RasWT and Raf-RBD-C3LS; and (c) to test MCAMD in prostate cancer cells.

15. SUBJECT TERMS
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Introduction

Effective controlling tumor growth and selectively killing tumor cells are the most important tasks in cancer therapies. Here we take a molecular approach to create a tumor cell killing-device: mitogenesis-coupled apoptosis molecular device (MCAMD). MCAMD is a molecular device that automatically senses tumorigenesis by monitoring cellular mitogenic process and induces apoptosis once cellular mitogenesis occurs. MCAMD contains two parts: one is tumorigenesis sensor portion and another is death execution portion. This proposal is to construct a Ras/Raf/caspase-3-based MCAMD, in which the activation of Caspase-3 is coupled with interaction of Ras and Raf. The main idea of Ras/Raf/caspase-3-based MCAMD is to fuse one subunit of caspase-3 to Raf and the other subunit to Ras. Once Ras interacts with Raf in response to mitogenic signal, the two subunits of caspase-3 that are fused to Ras and Raf will be brought together to form mature active caspase-3, thus cellular apoptosis will be initiated. The approach in this proposal is the first step of our long-term goal: developing MCAMD as an effective and selective therapeutic technique for cancers.

Body

1. Construction of MCAMD. The plasmids required in MCAMD were constructed by polymerase chain reaction (PCR) with Ras, Raf and Caspase-3 cDNAs as templates (Figure 1). The plasmids constructed for MCAMD are listed as following: (a) pcDNA3-Myc-RasG12V; (b) pcDNA3-Myc-C3SS-$(GGGGS)_2$-Ras; (c) pcDNA3-Myc-C3SS-$(GGGGS)_2$-RasG12V; (d) pcDNA3-HA-Raf-RBD-C3LS; (e) pcDNA3-HA-Raf-RBD-(GNNGGNGGS)-C3LS; (f) pTet- Myc-C3SS-$(GGGGS)_2$-Ras; (g) pTet- HA-Raf-RBD-(GNNGGNGGS)-C3LS; (h) pGEX4T3-C3LS; (i) pcDNA3-Myc-C3SS-$(GGGGS)_2$-Cdc42; (j) pcDNA3-Myc-C3SS-(GGGGS)2-Cdc42 Q61L. In the names, C3SS stands for Caspase-3 small subunit, C3LS for Caspase-3 large subunit, and RBD for Ras-binding domain. The amino acids in the parentheses are flexible linkers. RasG12V and Cdc42 Q61L are GTPase-defective mutants of small GTPases Ras and Cdc42, respectively.

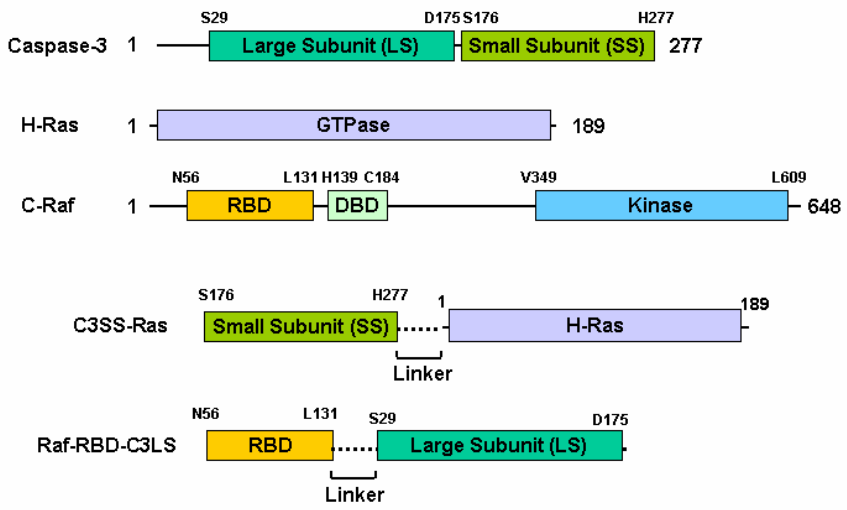

Figure 1. Construction of MCAMD. The schematic representation of Caspase-3, H-Ras, C-Raf, C3SS-Ras and Raf-RBD-C3LS. The numbers indicate amino acid residue positions. The letter before the number indicates the amino acid at the position. In C3SS-Ras and Raf-RBD-C3LS, the amino acid residue positions are labeled according to the numbers in original proteins. In Raf, RBD, Ras-binding domain; DBD, DAG-binding domain.

For engineering of MCAMD, the amino terminus of Ras is the only site that can be used for the engineering because the carboxyl terminus of Ras is required for the farnesylation. Therefore, we designed the structure of the Ras-caspase-3 and Raf-caspase-3 fusion proteins as shown in Figure 1: (i) the amino terminus of the large subunit (LS) of caspase-3 was connected to the carboxyl terminus of Raf-RBD, and (ii) the carboxyl terminus of the small subunit (SS) of caspase-3 was connected to the amino terminus of Ras. To reduce the conformational restrictions of caspase-3 subunits that are

caused by fusion to Ras or Raf, we added a flexible linker between Ras and the SS or Raf –RBD and the LS.

2. Results of testing MCAMD. After construction of the plasmids of MCAMD, we tested the idea of MCAMD: coupling the interaction of Ras/Raf, a general mitogenic biochemical process, to Caspase-3 activation and apoptosis. The initial experiments were performed in COS7 cells for high efficiency of transfection and expression of engineered proteins.

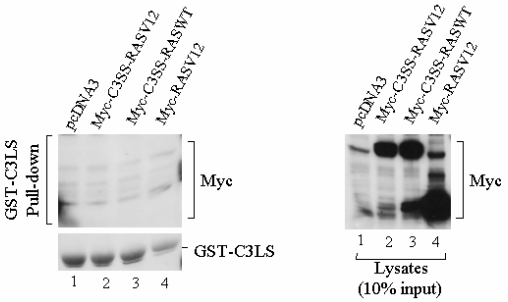

Figure 2. Separated C3SS and C3LS do not interact each other. GST-C3LS-bound beads were incubated with pcDNA3 (vector), Myc-tagged C3SS-RasG12V, C3SS-RasWT, and RasG12V transfected COS7 cell lysates (the right figure). The co-precipitated proteins were immunoblotted with anti-Myc (the left top panel). The loaded bead-bound GST-C3LS is shown in the left bottom panel by coomassie blue staining.

The basic principle for Ras/Raf-based MCAMD is to couple the interaction of activated Ras with Raf to formation of active Caspase-3 by fusing the small subunit of Caspase-3 to Ras and the large subunit of Caspase-3 to Raf-RBD. However, there is a possibility that the small subunit and the large subunit of Caspase-3 can directly interact each other to form mature Caspase-3 without the interaction of Ras and Raf-RBD. If this is the case, then MCAMD will not work. Therefore, the first step in testing MCAMD is to examine the interaction of the small subunit and the large subunit of Caspase-3. There are two methods for examination of the interaction: co-immunoprecipitation and GST-fusion protein pull-down. The co-immunoprecipitation, however, is not suitable for detection of a weak interaction. The GST-fusion protein pull-down can detect a very weak interaction due to excessive amount of fusion protein. Therefore, we applied GST-fusion protein pull-down to test if there is a direct interaction occurs between the C3SS and C3LS. We incubated GST-C3LS-bound beads with C3SS-Ras-expressed COS7 cell lysates (right panel, Figure 2), and detected the amount of C3SS-Ras co-precipitated with GST-C3LS-bound beads. As shown in the left top panel of Figure 2, we did not detect any C3SS-Ras co-precipitated with GST-C3LS, indicating that no interaction

between the C3SS and C3LS. These data suggest that separated C3SS and C3LS cannot form a mature Caspase-3 by themselves.

Next we tested the interaction of Ras and Raf-RBD. When we co-expressed Myc-tagged Ras GTPase defective mutant G12V with HA-tagged Raf-RBD-C3LS, we observed interaction of RasG12V with Raf-RBD-C3LS using co-immunoprecipitation assays, as we expected (lane 2, Figures 3A and 3B). This indicates that the RBD domain of Raf is functional and can binds to Ras. However, when we co-expressed Myc-tagged C3SS-RasG12V with HA-tagged Raf-RBD-C3LS, we did not observe any expression of the two proteins in cell lysates (lane 1, the bottom two panels, Figure 3A and 3B). This suggests that co-expression of C3SS-RasG12V and Raf-RBD-C3LS induced a rapid cell death, thus no expression of either protein could be detected. To ensure that expression of C3SS-RasG12V or Raf-RBD-C3LS did not have any defect, we expressed these two plasmids separately in COS7 cells. As shown in Figure 3C, the expression of Myc-C3SS-RasG12V or HA-Raf-RBD-C3LS alone was normal (lanes 1 and 3, figure 3C), while no co-expression of these two fusion proteins was detected (lane 2, figure 3C). These data strongly indicate that C3SS-RasG12V and Raf-RBD-C3LS cannot co-exist in cells, implicating apoptosis and rapid cell death occurred upon co-expression of C3SS-RasG12V and Raf-RBD-C3LS.

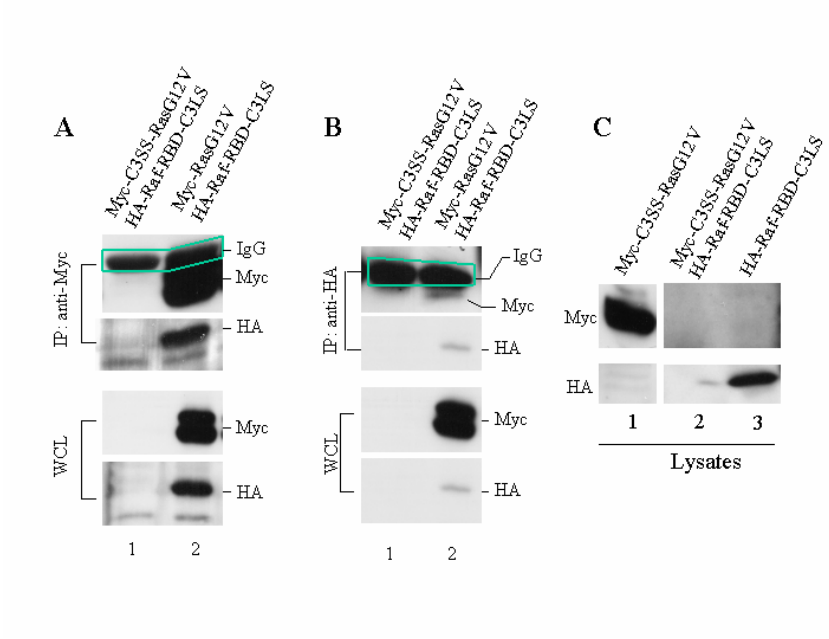

Figure 3 C3SS-RasG12V and Raf-RBD-C3LS cannot co-express in cells. Myc-tagged C3SS-RasG12V, RasG12V and HA-tagged Raf-RBD-C3LS were transfected or co-transfected into COS7 cells. Myc-tagged C3SS-RasG12V or RasG12V was immunoprecipitated by anti-Myc. HA-tagged Raf-RBD-C3LS was immunoprecipiated by anti-HA. The co-immunoprecipiated Raf-RBD-C3LS was detected by immunobloting with anti-HA (the second top panel in Figure **A**) and co-immunoprecipitated Myc-RasG12V was detected with anti-Myc (the top panel of Figure **B**). The expression of the proteins was shown in whole cell lysates (WCL) (Figure **C** and the two bottom panels in figures **A** and **B**).

We further determined whether co-expression of C3SS-Ras wild type (WT) and Raf-RBD-C3LS yields apoptosis and cell death. To reduce the effect of protein fusion on changing protein native conformation, we added a flexible linker $(GGGGS)_2$ between C3SS and Ras and (GNNGGNGGS) between Raf-BRD and C3LS, as shown in Figure 1 and Figure 4. We expected that C3SS-Ras WT and Raf-RBD-C3LS did not have interaction and would not produce apoptosis. However, similar to the results of co-expression of C3SS-RasG12V and Raf-RBD-C3LS (shown in Figure 2C), expression of each of chimera proteins alone was well detectable (lanes 2 and 3, figure 4) while no co-expression of C3SS-Ras WT and Raf-RBD-C3LS was detected (lane 1, figure 4). These data suggest that overexpressed wild type Ras may have a weak interaction with overexpressed Raf-RBD, and co-expression of C3SS-Ras WT and Raf-RBD-C3LS causes a rapid cell death. These data raise a question how to control activation of Ras/Raf-based MCAMD by mitogenic signaling.

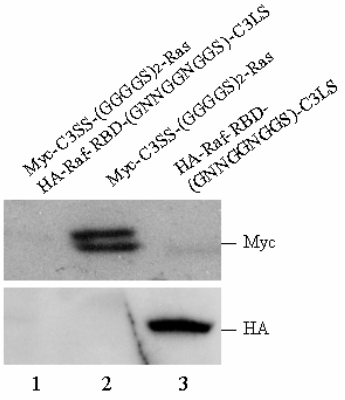

Figure 4. C3SS-RasWT does co-express with Raf-RBD-C3LS. Myc-tagged C3SS-(GGGGS)2-Ras WT (wild type) and HA-tagged Raf-RBD-(GNNGGNGGS)-C3LS were transfected or co-transfected into COS7 cells. The protein expression was detected by immunoblotting with anti-Myc (top panel) or anti-HA (bottom panel).

3. Future studies. The project has not been completed. There are many experiments need to be done to reach the goals proposed in the original grant proposal. (a) We need to overcome signaling switch problem of the MCAMD. It seems that overexpressed C3SS-Ras WT can interact with overexpressed Raf-RBD-C3LS, which results in constitutively active Caspase-3 formation. Alternative approach is to use tetracycline-inducible expression system to control the expression of C3SS-Ras WT and Raf-RBD-C3LS. Based on this consideration, we have cloned C3SS-RasWT and Raf-RBD-C3LS into a tetracycline-inducible expression vector. We have not tested the expression of the plasmids yet. (b) We need to assay caspase-3 activity and apoptosis upon co-transfection of

tetracycline-inducible expression of C3SS-RasWT and Raf-RBD-C3LS. (c) We need to test MCAMD in prostate cancer cells.

Key research accomplishments:

(1) We have successfully constructed Ras-Raf-based MCAMD. This is the first MCAMD ever built.

(2) We have confirmed that separated C3SS and C3LS do not interact each other, thus will not form a mature Caspase-3 by themselves. This establishes a base for Caspase-3 in application for construction of MCAMD.

(3) We have found that C3SS-Ras and Raf-RBD-C3LS could express separately, however, could not co-express in COS7 cells, implicating that co-expression of C3SS-Ras and Raf-RBD-C3LS might cause a rapid cell death. This suggests that MCAMD is workable.

MCAMD is a novel concept in cancer therapy. Because MCAMD specifically attacks mitogenic or tumorigenic cells, MCAMD has a great potential to become a more effective and selective means for cancer therapy than a conventional treatment. Furthermore, The concept of MCAMD can be extended beyond cancer therapy. Any disease that has a unique biochemical event can be treated with a similar molecular device to MCAMD.

Reportable outcomes:

Through this research we have acquired experience in construction of MCAMD, testing of MCAMD, and trouble-shooting of MCAMD, and built a foundation for exploration of the application potential of MCAMD for cancer therapy. We will follow this research and continue to work on MCAMD and eventually construct a workable MCAMD for cancer therapy.

Conclusions

Effective and specific cancer therapeutic techniques are urgent tasks for cancer cure. During tumorigenesis, primary tumorigenic factor usually induces multiple secondary malignant processes, such as mutations and abnormality of biochemical signaling, and makes tumors very difficult to cure. Therefore, specific induction of apoptosis of tumor cells is a critical outcome for an effective cancer therapeutic technique. Our studies are based on this idea and attempt to create artificial molecules that can sense tumorigenesis and couple tumor-specific biochemical reactions to Caspase-3 activation and apoptosis. Such artificial molecules are named as mitogenesis-coupled apoptosis molecular device (MCAMD). In this grant proposal, we proposed to construct Ras/Raf-based MCAMD, i.e. fuse one subunit of caspase-3 to Raf and the other subunit to Ras. Once Ras interacts with Raf in response to mitogenic signal, the two subunits of caspase-3 that are fused to Ras and Raf will be brought together to form mature active caspase-3, thus cellular apoptosis will be initiated.

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References: None

Appendices:

(1) A list of Personnel receiving pay from the research effort:

Wannian Yang, Ph.D., Staff Scientist (5% effort)
Qiong Lin, Ph.D., Research Scientist II (100% effort)